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(54) Title: REGULATING ANIMAL REPRODUCTION

(57) Abstract

A contraceptive veterinary vaccine including (a) a protein-hormone conjugate of a luteinizing hormone (LH), analogue thereof, fragment thereof or derivative thereof, or a follicle stimulating hormone (FSH), analogue thereof, fragment thereof, or derivative thereof, and (b) a protein-hormone conjugate of a luteinizing hormone releasing hormone (LH-RH), analogue thereof, fragment thereof, or derivative thereof.

REGULATING ANIMAL REPRODUCTION

The present invention relates to a method of regulating the reproductive function of animals and to a veterinary composition for use in such a method.

It is known in the prior art to regulate reproductive functions in animals in a variety of ways. Artificial and natural products such as prostaglandins, pregnant mare serum gonadotrophin, melatonins and the like have been proposed for regulation of reproduction in animals. However, such treatments have proved to be of limited value in reduced or suppressing ovulation in female animals in particular. In relation to male animals, surgical castration is still the preferred contraceptive technique. However, there are a number of disadvantages associated with castration, including possible haemorrhage, infection, weight loss and reduced growth rate.

It has recently been proposed to utilise immunisation against luteinising hormone releasing hormone (LH-RH) to inhibit reproduction function in animals. However, variability of response and side effects associated with the use of potent adjuvants (absesses, granulomas) have been noted in several species of animals.

Further, transient and variable effects on testicular and ovarian function have been observed and reported in the prior art. For example, production of antibodies against LH-RH has been reported in the male and female rat, rabbit, dog, monkey, sheep, cattle and horses (Shanbacher B.D., Active immunization against LH-RH in the male; Jeffcoate I.A., Keeling, B.K., Active immunization against LH-RH in the female, 1984. In: Immunological

aspects of reproduction in mammals, ed. D.B. Crighton, Butterworths).

Moreover, in these experiments the effects of LH-RH immunization on a reproductive function are temporary and related to the LH-RH antibody titres. LH-RH immunization is not equally effective in all species of animals and a large proportion of immunized animals fail to respond with an effective suppression of reproductive function. Ineffectiveness of LH-RH immunization as a replacement for surgical desexing is especially evident in cattle (Schanbacher 1984).

LH-RH by itself is not antigenic because of its small size (approx. 1200 daltons) and, in order to obtain antibodies against it, it is necessary to attach LH-RH to much larger natural or synthetic carrier molecules. Numerous techniques for attachment of LH-RH to various carrier molecules are known including glutaraldehyde condensation, diisocyanate toluene, benzidine derivatives and carbodiimide. These techniques are not easy to control and it is very difficult to obtain conjugates of a predictable composition and of consistent quality. The most widely used technique for LH-RH conjugation to the carrier molecules is carbodiimide reaction and this reaction is particularly unpredictable (Schanbacher 1984). The inconsistent quality of conjugates and unpredictable configuration of created antigen molecules contribute to the difficulties of obtaining sufficient titres of specific antibodies to neutralize circulating endogenous hormones.

Similarly inconsistent results have been obtained with immunization against pituitary hormone lutenizing

hormone (LH). Schanbacher (1985) Theriogenology 24:59. "Effects of Active Immunization of the Ram and Bull against Luteinizing Hormone" showed that immunization against ovine LH produced castration-like response in young bulls but was not effective in ram-lambs. Immunization against yet another reproductive hormone-testosterone (T) produces, paradoxically, excessively high levels of circulating testosterone and in addition a high degree of immune complex nephritis is observed. (N.B. Haynes and J.A. Southee, Effects of immunization against steroid hormones in male endocrinology, 1984. In: Immunoligical aspects of reproduction in mammals, ed. D.B. Crighton, Butterworths).

Accordingly, it is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties relating to the prior art.

Accordingly, in a first aspect of the present invention there is provided a contraceptive veterinary vaccine including

(a) a protein hormone conjugate of a luteinizing hormone (LH), analogue thereof, fragment thereof or derivative thereof, or a follicle stimulating hormone (FSH) analogue thereof, fragment thereof,

or derivative thereof, and

(b) a protein hormone conjugate of a luteinizing hormone releasing hormone (LH-RH), analogue thereof, fragment thereof, or derivtive thereof.

It has been found that the separate manipulation of a single element of the reproductive regulatory mechanisms is not capable of providing an

effective desexing technology for various species of animals. Immunization against at least two interdependent components of reproductive feedback loop is an effective desexing vaccine for different species of animals. Immunization against two elements of reproductive control mechanisms provides a substantially fail-safe mechanism.

Components (a) and (b) of the contraceptive vaccine may be present in any suitable relative amounts. The weight ratio of (a) to (b) may range from approximately 1:2 to 2:1.

Techniques utilising immunisation against a single element of reproductive control mechanism (e.g. LH-RH) in order to be effective should be substantially 100% effective in all individuals. There is ample evidence that it is not possible to achieve this level of control via a single element. However, the contraceptive vaccine according to the present invention utilizing immunization against two interdependent hormones (e.g. LH-RH and LH) needs to be only partially successful in evoking an immune response to each hormone to effectively block or interrupt reproductive function. Until recently immunization of animals against pituitary hormones was not commercially feasible because of difficulties in supplying sufficient quantities of the hormones and their price. The recombinant DNA techniques changed the situation and these hormones or their fragments can be producted in large quantities and at a low cost.

The contraceptive vaccine according to the present invention may be utilised with any animal species. Animal species including cattle, sheep, goats, cats, guinea pigs, pigs, dogs, reindeer, horses and primates may be so treated. The contraceptive vaccine is particularly applicable to

domestic pets such as dogs and cats.

The hormone-protein conjugates may be formed utilising conventional techniques. For example, heterobifunctional agents such as SPDP, carbodiimide, glutaraldehyde or biotin/avadin systems may be used. Preferably, the hormone-protein conjugates are formed utilising a methodr which is both repeatable and predictable. Because of the repeatability and predictability this technique is particularly suited for large scale production. Creation of properly structured antigens presenting always the same antigenic site to the immunosystem produces a more uniform immune response in the animals. Specifically, as the protein carrier, tetanus toxoid (TT) is preferred. The protein carrier is activated with 6-maleimido caproic acyl N-hydroxy succinimide ester (MCS) to introduce maleimido reactive groups. For example, if the maleimido reactive groups are introduced in a ratio of approximately 30 per 100,000 daltons, a desired number of binding sites for the peptide hormones is created. The peptide hormones may in turn be activated by thiolation. Thiolation may be achieved by reaction with, e.g. N-acetyl homocysteine thiolactone (AHTL).

In a preferred form of this aspect of the present invention there is provided a contraceptive vaccine as described above further including

(c) at least one adjuvant for the contraceptive vaccine.

The at least one vaccine adjuvant may be selected from aluminium hydroxide, Freund's Incomplete Adjuvant, Fruend's Complete Adjuvant, DEAE dextran,

levamisole, PCG and polyA polyC or polyU. However, in a preferred form, the vaccine adjuvant includes a cell wall immunostimulant or mixturs thereof. The cell wall immunostimulant may be a cell wall fraction of a mycobacterium phlei or smegmatis.

This fraction may be obtained by lysosome digestion of purified mycobacterial cell walls and is capable of replacing, at least in part, standard adjuvants such as described above, in particular the most commonly used, Fruend's Complete Adjuvant. The cell fraction is body tissue compatable, stimulate immune response to the viral and protein antigens and also does not induce sensitivity to tuberculin. These features make the mycobacterial cell wall preparation eminently suitable for formulating the vaccines for companion and food producing animals.

In accordance with a further aspect of the present invention there is provided a method of inhibiting the reproductive functions of animals which method includes providing a contraceptive vaccine including

(a) a protein-hormone conjugate of a luteinizing hormone (LH), analogue thereof, fragment thereof or derivative thereof, or a follicle stimulating hormone (FSH) analogue thereof, fragment thereof or derivative thereof, and

(b) a protein-hormone conjugate of a luteinizing hormone releasing hormone (LH-RH), analogue thereof, fragment thereof, or derivative thereof;

and administering an effective amount of the vaccine to the animal to be treated.

The method of inhibiting the reproductive functions of animals may include preventing or suppressing ovulation and/or oestrous cyclicity in female animals and prevention or suppression of sexual behaviour in male animals.

The vaccine may be administered parenterally. Parenteral administration may include subcuaneous, intramuscular or intravenous injeciton, oral administration or adsorption through the skin or by mini pump either implanted in the animal or attached to the hide of the animal.

The dose rates effective will vary with the weight and species of animal. Optimum dose rates for individual species may be selected utilising simple experimentation. However, as a guide for small animals such as domestic dogs or cats each dose may include from approximately 200 to 300 microgram of the luteinising hormone or follicle stimulating hormone conjugate and from approximately 200 to 300 microgram of luteinizing hormone releasing hormone conjugate. Where a vaccine conjugate is used this may be present in amounts of from approximately 150 to 250 micrograms.

Preferably a single vaccination is only required but a second vaccination may be undertaken for security.

The present invention will now be more fully described with reference to the accompanying example. It should be understood, however, that the following description is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLE

Preparation of Vaccine:

Lyophilized cell wall immunostimulant (Raglan

Research, USA) was mixed with the lyophilized hormone-TT conjugates. The ratio of immunostimulant and conjugates was such that each injection contained 200 ug of immunositmulant and 250 ug of each conjugate. The mixture was then combined with a small quantity of Marcol-82 oil (1% of total volume) and emulsified by sonication with buffered physiological saline containing 0.5% of Tween-80 (polyoxyethylene sorbitan mono-oleate).

Hormones

1. L-Lys^8-LH-RH (Luteinzing Hormone Releasing Hormone) analogue was purchased from Peninsula Laboratories, USA.
2. LH (Luteinizing Hormone) and FSH (Follicle Stimulating Hormone). Ovine hormones (NIH-FSH-S12 and NIH-LH-S18) were obtained from the National Hormone and Pituitary Programme USA.

Preparation of Conjugates:

The conjugates of carrier protein and hormones were prepared according to Lee et al. (1980) Molecular Immunology 17: 749-756. "A method for preparing β-hCG COOH peptide-carrier conjugates of predictable composition". As a carrier-tetanus toxoid (TT) was chosen. Commercial TT (CSL) was further purified and concentrated by gel filtration of Bio-Gel P-60. Briefly - the protein carrier (TT) was reacted with 6-maleimido caproic acyl N-hydroxy succinimide ester (MCS) to introduce maleimido reactive groups in a ratio of approximately 30 per 100,00 daltons. By regulating the molar ratio of MCS in relation to the carrier protein a desired number of binding sites for peptide hormones containing thiol groups (cysteine residues) could be created.

L-Lys^8-LH-RH was thiolated by reaction with

N-acetyl homocysteine thiolactone (AHTL). The MCS modified carriers and peptides containing a thiol group were conjugated as follows: MCS-modified TT was dissolved in a small volume of N_2-saturated 0,1M sodium-phosphate-0,1M EDTA pH. 6.6 buffer. This solution was added to the reaction vial containing an amount of dry peptide in excess of the molar equivalent of maleimido groups in the carrier. The reaction was conducted in the nitrogen atmosphere at room temperature overnight. The conjugate was purified on Seph G-25 column equilibrated in 0,2M NH_4HCO_3 buffer. The conjugate eluted in void volume was lyophilised.

Animals and Immunization

Six virgin Merino ewes regularly cycling every 17 days were observed for 5 cycles prior to immunization. The ewes were chosen from a 50-animal flock of controls. The animals were kept with a teaser ram fitted with Siro-Sine harness. Experimental animals were immunized with the mixture of conjugates and the immunostimulant emulsified in phosphate buffered saline. The two 1ml intramuscular injections were given 21 days apart. The first injection was given at the detection of oestrus.

Results:

The regular cycling activity stopped after the first injection of conjugates. The oestrus was not detected by the teaser ram in immunized animals over a 12 months' observation period. It appears that the second injection was helpful in prevention of reproductive activity in the ewe. Reproductive activity was abolished in all immunized animals for over 12 months regardless of individual responses to one or other conjugate. Presence of antibodies to both conjugates - even

though with relatively low titres was enough to disrrupt reproductive activity of the ewe. (Fig. 1 and Fig. 2).

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

CLAIMS

1. A contraceptive veterinary vaccine including

(a) a protein-hormone conjugate of a luteinizing hormone (LH), analogue thereof, fragment thereof or derivative thereof, or a follicle stimulating hormone (FSH) analogue thereof, fragment thereof, or derivative thereof, and

(b) a protein-hormone conjugate of a luteinizing hormone releasing hormone (LH-RH), analogue thereof, fragment thereof, or derivative thereof.

2. A contraceptive veterinary vaccine according to claim 1 wherein the weight ratio of protein-hormone conjugate (a) to protein-hormone conjugate (b) is in the range of approximately 1:2 to 2:1.

3. A contraceptive veterinary vaccine according to claim 2 including a protein-hormone conjugate of a luteinizing hormone and a protein-hormone conjugate of a luteinizing hormone releasing hormone.

4. A contraceptive veterinary vaccine according to claim 2 including a protein-hormone conjugate of a follicle stimulating hormone and a protein-hormone conjugate of a luteinizing hormone releasing hormone.

5. A contraceptive veterinary vaccine according to claim 2 wherein the protein is a tetanus toxoid.

6. A contraceptive veterinary vaccine according to claim 5 wherein the tetanus toxoid protein is activated with 6-maleimido caproic acyl N-hydroxy succinimide ester.

7. A contraceptive veterinary vaccine according to claim 6 wherein the hormone component of each of the protein-hormone conjugates is activated by thiolation.

8. A contraceptive veterinary vaccine according to claim 7 wherein the hormones are activated with N-acetyl

homocysteine thiolactone.

9. A contraceptive veterinary vaccine according to claim 2 further including

(c) at least one adjuvant for the contraceptive veterinary vaccine selected from aluminium hydroxide, Freund's Incomplete Adjuvant, Freund's Complete Adjuvant, DEAE dextran, levamisole, PCG and polyA polyC or polyU, a cell wall immunostimulant or mixtures thereof.

10. A contraceptive veterinary vaccine according to claim 9 wherein the at least one adjuvant includes a cell wall immunostimulant selected from a cell wall fraction of a mycobacterium phlei or smegmatis.

11. A method of preparing a contraceptive veterinary vaccine including providing an effective amount of a luteinizing hormone releasing hormone, analogue thereof, fragment thereof or derivative thereof; and a luteinizing hormone, analogue thereof, fragment thereof or derivative thereof, or a follicle stimulating hormone, analogue thereof, fragment thereof or derivative thereof; and an effective amount of a protein carrier; reacting a portion of the protein carrier with the luteinizing hormone releasing hormone to form a first protein-hormone conjugate; reacting a portion of the protein carrier with the luteinizing hormone or follicle stimulating hormone to form a second protein-hormone conjugate; and mixing the conugates so formed.

12. A method according to claim 11 wherein the protein is a tetanus toxoid.

13. A method according to claim 12 wherein the tetanus toxoid is activated with 6-maleimido caproic acyl N-hydroxy succinimide ester.

14. A method according to claim 13 wherein each of the peptide hormones are activated by thiolation.

15. A method according to claim 12 further including providing at least one adjuvant for the contraceptive veterinary vaccine selected from aluminium hydroxide, Freund's Incomplete Adjuvant, Freund's Complete Adjuvant, DEAE dextran, levamisole, PCG and polyA polyC or polyU, a cell wall immunostimulant or mixtures thereof; and mixing the at least one adjuvant with the mixture of protein-hormone conjugates.

16. A method of inhibiting the reproductive functions of animals which method includes providing a contraceptive veterinary vaccine including

(a) a protein-hormone conjugate of a luteinizing hormone (LH), analogue thereof, fragment thereof or derivative thereof, or a follicle stimulating hormone (FSH) analogue thereof, fragment thereof, or derivative thereof, and

(b) a protein-hormone conjugate of a luteinizing hormone releasing hormone (LH-RH), analogue thereof, fragment thereof, or derivative thereof;

and administering an effective amount of the vaccine to an animal to be treated.

17. A method according to claim 16 wherein the inhibitions of the reproductive functions of animals includes preventing or supressing ovulation and/or oestrous cyclicity in female animals or prevention or suppression of sexual behavious in male animals.

18. A method according to claim 17 wherein the weight ratio of protein-hormone conjugate (a) to protein-hormone conjugate (b) is in the range of approximately 1:2 to 2:1.

19. A method according to claim 18 including a protein-hormone conjugate of a luteinizing hormone and a protein-hormone conjugate of a luteinizing hormone releasing hormone.

20. A method according to claim 18 including a protein-hormone conjugate of a follicle stimulating hormone and a protein-hormone conjugate of a luteinizing hormone releasing hormone.

21. A method according to claim 18 further including

(c) at least one adjuvant for the contraceptive veterinary vaccine selected from aluminium hydroxide, Freund's Incomplete Adjuvant, Freund's Complete Adjuvant, DEAE dextran, levamisole, PCG and polyA polyC or polyU, a cell wall immunostimulant or mixtures thereof.

FIGURE 1

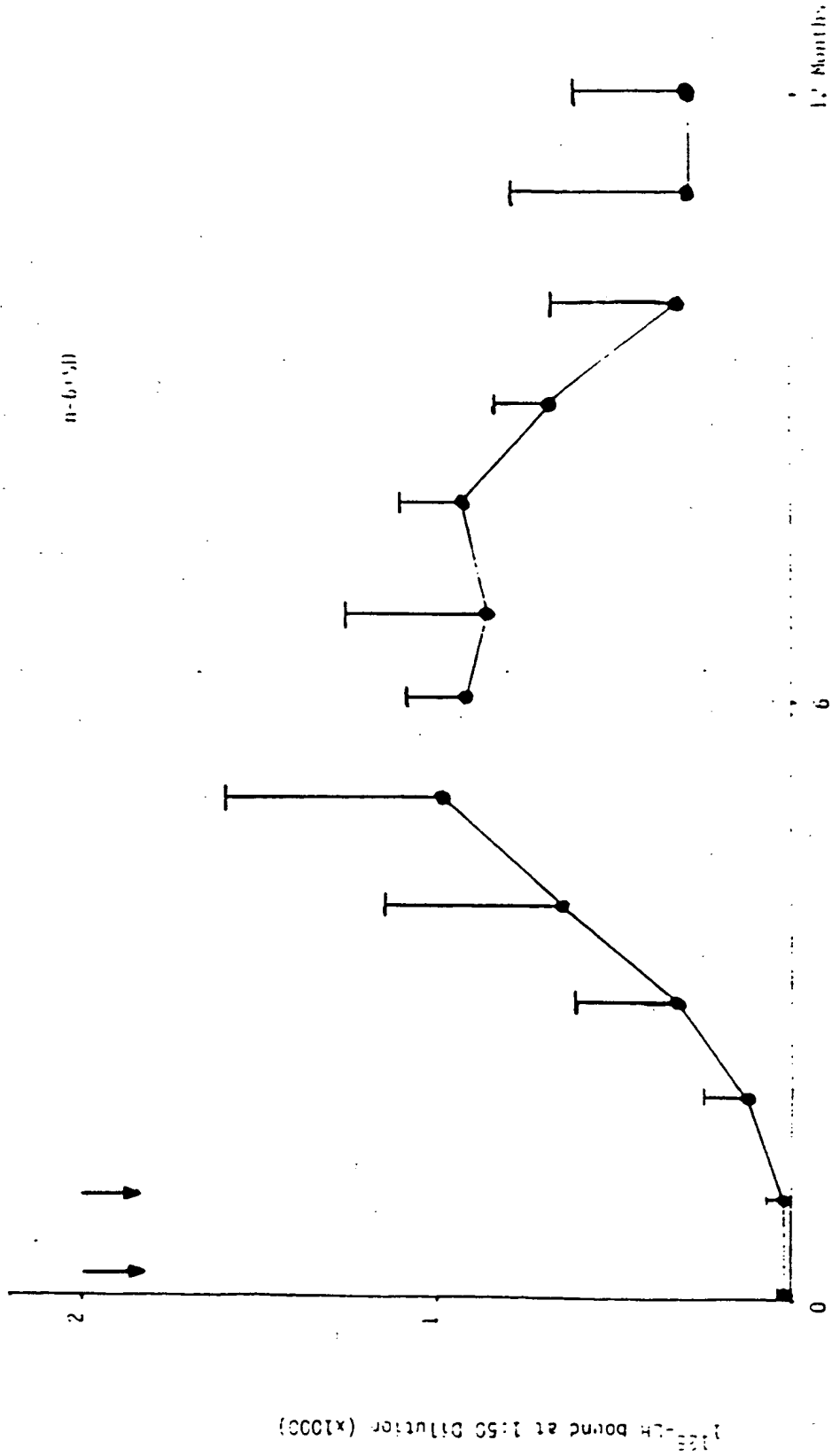

FIGURE 2

ANTIBODY RESPONSE IN SHEEP AFTER IMMUNIZATION WITH TT CONJUGATE

SUBSTITUTE

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00241

I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] A61K 39/385, 37/43, 37/38

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
IPC	A61K 37/38, 37/43

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	AU,B, 80826/75 (503647) (ALL INDIA INSTITUTE OF MEDICAL SCIENCES) 11 November 1976 (11.11.76)	(1-21)
A	AU,A, 52886/79 (VEB BERLIN-CHEMIE) 3 July 1980 (03.07.80)	(1-21)
A	US,A, 4673665 (HOECHST) 16 June 1987 (16.06.87)	(1-21)
A	EP,A, 136781 (AMERICAN HOME PRODUCTS CORPORATION) 10 April 1985 (10.04.85)	(1-21)
A	GB,A, 1547557 (AMERICAN HOME PRODUCTS CORPORATION) 20 June 1979 (20.06.79)	(1-21)

* Special categories of cited documents:
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of the International Search Report
4 November 1987 (04.11.87)	(12.11.87) 12 NOVEMBER 1987
International Searching Authority: Australian Patent Office	Signature of Authorized Officer: J.P. PULVIRENTI

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00241

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
AU 52886/79	BG 33773	DE 2942500	DK 5450/79
	FR 2445148	GB 2044613	IT 1119945
	JP 55108822	SE 7910548	US 4255420
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	EP 83925	FI 830040	NO 830041
	NZ 202978	ZA 8300093	
EP 136781	BE 900332	DE 3429261	JP 60120820
	ZA 8405550		
GB 1547557	US 4338305	US 4272432	

END OF ANNEX